Cholesterol Sulfate Inhibits Proteases that are Involved in Desquamation of Stratum Corneum

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We previously reported that desmosomes play a key role in the adhesion of corneocytes, and their digestion by two types of serine proteases leads to desquamation. Patients with recessive X-linked ichthyosis show hyperkeratosis attributable to desmosomes, associated with an increased content of cholesterol sulfate (CS) and an increased thickness of stratum corneum. In this study, therefore, we examined the possibility that CS provokes the abnormal desquamation, acting as a protease inhibitor. Scaling was induced on mice after topical application of chymostatin and leupeptin. Visible scale was also observed on mice after topical application of CS. We found that the stratum corneum thickness of CS-treated mice was increased in comparison with that of vehicle-treated mice. The thickness of the epidermis and the labeling index with proliferating cell nuclear antigen from CS-treated mice was almost the same as that from vehicle-treated mice. Moreover, in the stratum corneum of CS-treated mice, the content of desmosomes was higher than that in vehicle-treated mice. CS also inhibited the protease-induced cell dissociation of human stratum corneum sheets. *In vitro*, CS competitively inhibited both types of serine protease: the K_i for trypsin was 5.5×10^{-6} M and that for chymotrypsin was 2.1×10^{-6} M. These results indicate that CS retards desquamation by acting as a protease inhibitor. Thus, accumulation of stratum corneum in recessive X-linked ichthyosis may be a result of the inhibition by excessive CS of proteases involved in the dissolution of desmosomes, required for desquamation of the stratum corneum. *Key words: chymotrypsin/desmosome/ichthyosis/trypsin. J Invest Dermatol 111:189–193, 1998*

Recessive X-linked ichthyosis, which is caused by a deficiency of steroid sulfatase, is a disease exhibiting hyperkeratosis accompanied by accumulation of cholesterol sulfate (CS). It has long been thought that lipids, especially CS, act as corneocyte cohesion elements. Cholesterol-lowering substances are known to induce scaling disorder (Williams and Elias, 1987; Williams *et al*, 1987), whereas topical application of CS induced scaling without acanthosis or increased labeling index (Maloney *et al*, 1984; Elias *et al*, 1984). The highly cohesive ungulate hoof is particularly rich in CS (Wertz and Downing, 1984); however, no significant differences in CS content were found between tightly cohesive stratum corneum of the palm, and the loosely cohesive stratum corneum of the upper arm (Serizawa *et al*, 1992). Thus, the relationship between CS and accumulated stratum corneum is unclear. Williams suggested the possibility that the failure to desquamate in recessive X-linked ichthyosis may be due to CS-mediated inhibition of desmosome proteolysis (Williams, 1991). We have investigated the mechanism of desquamation in stratum corneum, showing that desmosomes play a key role in the adhesion of corneocytes, and that their digestion by two types of serine proteases leads to desquamation (Suzuki *et al*, 1993, 1994). Lundström and Egelrud also reported that chymotrypsin-like enzyme activity in the stratum corneum may play a role in the desquamation process (Lundström and Egelrud, 1991; Sondell *et al*, 1995). It was reported that numerous desmosomal structures remained in the outermost layers of the stratum corneum in recessive X-linked ichthyosis (Anton-Lamprecht, 1974; Bazex *et al*, 1978; Mesquita-Guimarães, 1981). Because CS inhibited the serine protease, acrosin, required for normal sperm capacitation (Burck and Zimmerman, 1980), in this study we examined the possibility that CS acts as a protease inhibitor, retarding desquamation of stratum corneum.

MATERIALS AND METHODS

Animals and topical application Cholesterol 3-Sulfate (CS, Sigma, St. Louis, MO) and chymostatin (Peptide Institute, Osaka, Japan) were dissolved in dimethyl sulfoxide (DMSO, Wako, Japan) to prepare a 10 mM solution. Leupeptin (Peptide Institute) was dissolved in distilled water to prepare a 10 mM solution. Eighty microliters of CS solution, chymostatin and leupeptin, DMSO and distilled water, or DMSO alone was applied to the backs of hairless mice (Hos:HR-1) once a day. Six to 8 wk old male hairless mice were obtained from Hoshino (Saitama, Japan). After 3 d, biopsy and collection of stratum corneum by tape-stripping was undertaken.

Histologic observations Biopsy samples were fixed in 10% formalin and embedded in paraffin. The sections were stained with hematoxylin and eosin. Measurements of the thickness of the epidermis were made with a light microscope equipped with a CCD camera and image analysis system (Olympus XL-10, Japan). The sections were stained with proliferating cell nuclear antigen and positive cells were counted.

Electron microscopy Biopsy samples were fixed in half-strength Karnovsky's fixative, divided, and processed through reduced 1.0% osmium tetroxide followed by embedding in an Epon-epoxy mixture. Ultrathin sections were viewed in an electron microscope (H7100, Hitachi, Tokyo, Japan) after further contrasting in lead citrate and uranyl acetate. The number of stratum corneum layers was counted in a blinde manner.

Detection of desmosomes in the stratum corneum Desmosomal proteins were extracted from tape-stripped stratum corneum in a buffer containing

Manuscript received July 1, 1997; revised February 20, 1998; accepted for publication February 26, 1998.

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Abbreviation: CS, cholesterol sulfate.

0.1 M Tris HCl pH 9, 9 M urea, 2% sodium dodecyl sulfate, and 1% mercaptoethanol (200 µl of buffer per 2 mg stratum corneum) for 15 h at 37°C (Lundstrom and Egelrud, 1990). The extracts were prepared by mixing them with a Laemmli's sample buffer (Laemmli, 1970), followed by heating on a boiling water bath for 10 min. After centrifugation, the supernatant was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% gel. Electrophoretic transfer of proteins from the gel to PVDF membrane (Applied Biosystems, CA) was followed by immunostating with a Promega Protoblot Western Blot AP System (Promega, Madison, WI). Monoclonal antibody, anti-DG I, obtained by Boehringer (Mannheim, Germany), was used for detection of desmosomal glycoprotein, desmoglein I.

Cell dissociation from the stratum corneum sheet The stratum corneum sheet was obtained from the back of a volunteer 5 d after sun exposure. One milligram of strateum corneum sheet was incubated in 1 ml detergent mixture (8 mM DMDAO and 2 mM sodium dodecyl sulfate) with CS, phosphatidylcholine (Sigma, St. Louis, MO), palmitic acid (Sigma), taurocholic acid (Sigma), or each vehicle, containing 60 µg kanamycin at 37°C for 24 h (Takahashi *et al*, 1987). The number of released cells in the detegent mixture was counted using a Burker-Turk hemocytometer.

Assay for inhibitory behavior of cholesterol sulfate Crystalline porcin pancreatic trypsin (Wako, Osaka, Japan) and crystalline bovine pancreatic chymotrypsin (Sigma) were used to examine the inhibitory behavior of CS. Trypsin activity or chymotrypsin activity was examined by using Boc-Phe-Ser-Arg-MCA (3107-V) or Suc-Leu-Leu-Val-Tyr-MCA (3120-V) (Peptide Institute), respectively, as the substrate. Inhibitory activities of phosphatidylcholine, palmitic acid, cholesterol, and taurocholic acid were also examined. All assays were performed at 37°C in 0.1 M Tris HCl (pH 8.0).

Statistics The significance of difference was tested using the unpaired Student's t test.

RESULTS

Application of CS increases the stratum corneum thickness and induces abnormal scaling Visible scales were observed on the backs of mice 3 d after topical application of CS (**Fig 1**). Biopsy taken at this point showed increased thickness of the stratum corneum layer from mice treated with CS in comparison with that from vehicle-treated mice (**Fig 2**). Moreover, quantitative studies showed an increased number of stratum corneum layers of CS- *versus* vehicle-treated skin (**Fig 3**). Furthermore, the thickness of the living part of the epidermis from CS-treated mice was the same as that from vehicle-treated mice (data not shown). Finally, there were no differences between CS-treated and vehicle-treated skin in the labeling index with proliferating cell nuclear antigen, a measure of proliferative acitivity (data not shown). These results show the effect of CS on increasing the number of cell layers, the thickness, and the abnormal scaling of the stratum corneum. To examine whether CS acts as a detergent to disrupt enzyme function, we also applied phosphatidylcholine as amphopatic lipid to the backs of mice. Both daily treatment of 4.88 mg CS per ml (10 mM) and daily treatment of 0.488 mg CS per ml (1 mM) induced scales on the backs of mice after topical application; however, topical application of 4.88 mg phosphatidylcholine per ml did not show an increase in the number of scales compared with vehicle treatment (data not shown).

The increased content of desmosomal protein in the stratum corneum of CS-treated mice The stratum corneum of mice treated with CS or vehicle for 3 d was obtained by tape-stripping and the extracted proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred to PVDF membranes and reacted with anti-DG I. As seen in **Fig 4**, the tendency of the increased content of desmoglein I was noted in the stratum corneum of CS-treated mice ($p = 0.050$, $n = 3$). These results suggest that degradation of desmosomes was inhibited by the topical application of CS.

CS inhibited cell dissociation from the stratum cornuem sheet CS inhibited cell dissociation in this assay in a concentration-dependent manner (**Fig 5**). One hundred micromoles and 1 mM of CS inhibited cell dissociation to 64.0% and 56.9% of the 100% controls, respectively. Other amphopathic lipids, 1 mM phosphatidylcholine,

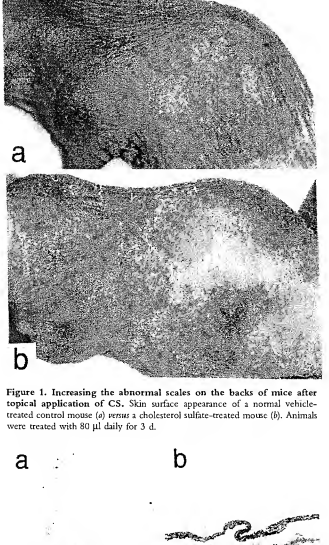

Figure 1. Increasing the abnormal scales on the backs of mice after topical application of CS. Skin surface appearance of a normal vehicle-treated control mouse (*a*) *versus* a cholesterol sulfate-treated mouse (*b*). Animals were treated with 80 µl daily for 3 d.

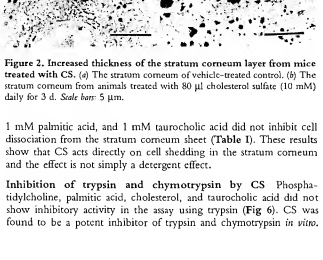

Figure 2. Increased thickness of the stratum corneum layer from mice treated with CS. (*a*) The stratum corneum of vehicle-treated control. (*b*) The stratum corneum from animals treated with 80 µl cholesterol sulfate (10 mM) daily for 3 d. *Scale bars*: 5 µm.

1 mM palmitic acid, and 1 mM taurocholic acid did not inhibit cell dissociation from the stratum corneum sheet (**Table I**). These results show that CS acts directly on cell shedding in the stratum corneum and the effect is not simply a detergent effect.

Inhibition of trypsin and chymotrypsin by CS Phosphatidylcholine, palmitic acid, cholesterol, and taurocholic acid did not show inhibitory activity in the assay using trypsin (**Fig 6**). CS was found to be a potent inhibitor of trypsin and chymotrypsin *in vitro*.

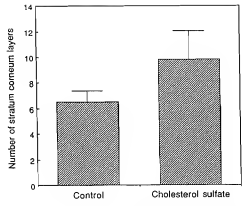

Figure 3. Application of cholesterol sulfate resulted in an increased number of stratum corneum layers as compared with vehicle treatment (n = 6, mean ± SD). Cholesterol sulfate *versus* control, $p < 0.01$.

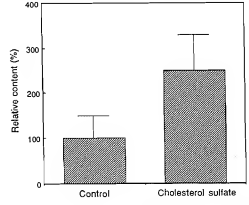

Figure 4. The level of anti-DG I-reactive protein in cholesterol sulfate-treated mice was higher than that of vehicle-treated mice (n = 3, mean ± SD).

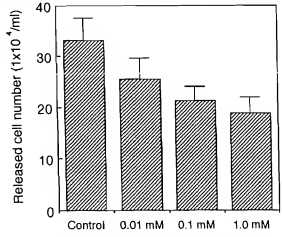

Figure 5. CS inhibited cell dissociation from the stratum corneum sheet. The stratum corneum sheet (1 mg) was incubated in a detegent mixture for 24 h at 37°C with cholsterol sulfate (n = 3, mean ± SD). Significant differences: 100 μM cholesterol sulfate *versus* control, $p < 0.05$; 1 mM cholesterol sulfate *versus* control, $p < 0.05$.

A double reciprocal plot showed that the inhibition of trypsin by CS is competitive (**Fig 7*a***), and a Dixon plot gave a K_i value of 5.5×10^{-6} M (**Fig 7*b***). Similar procedures showed that CS is also a competitive inhibitor of chymotrypsin with a K_i value of 2.1×10^{-5} M (**Fig 8*a*, *b***).

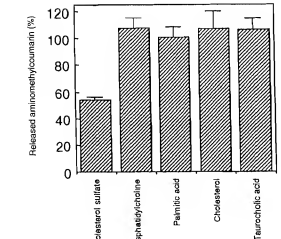

Figure 6. CS inhibited trypsin in the enzyme assay system *in vitro*. Two micromoles of CS, 2 μM of phosphatidylcholine, 2 μM of palmitic acid, 2 μM of cholesterol, and 2 μM of tauroric acid were examined for inhibitory activity using porcin pancreatic trypsin (n = 3, mean ± SEM). Significant difference: cholesterol sulfate *versus* control, $p < 0.05$.

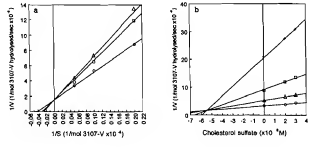

Figure 7. Cholesterol sulfate is a competetive inhibitor of trypsin. (*a*) Linewesver–Burk plot of the inhibition of trypsin by cholesterol sulfate (△, 3×10^{-7} M; □, 2×10^{-7} M); ○, no cholesterol sulfate. (*b*) Dixon plot of trypsin by cholesterol sulfate (○, 2.0×10^{-5} M 3107-V; △, 1.0×10^{-5} M 3107-V; □, 5.0×10^{-6} M 3107-V; +, 2.5×10^{-6} M 3107-V).

Table I. One millimole of CS inhibited cell dissociation from stratum corneum sheet

Lipids	Relativity (%) of the control: Mean	SD	Significance *versus* control
1 mM cholesterol sulfate	56.9	9.55	$p < 0.05$
1 mM palmitic acid	105.8	14.49	n.s.
1 mM phosphatidylcholine	134.1	25.18	n.s.
1 mM taurocholic acid	89.4	5.70	n.s.

Chymostatin and leupeptin also induced scales on the backs of mice after topical application. It has been thought that CS has multiple functions, e.g., acting as a surfactant. To explore the possibility that CS acts as an protease inhibitor, we examined the effect of commercially available protease inhibitors *in vivo* by topical application on skin. Large scales were observed on the backs of mice after topical application of 10 mM chymostatin and 10 mM leupeptin (**Fig 9*a***). The degree of scale was less obvious than that of the case of CS. Scales were assessed by measurement of their area using a binary image (**Fig 9*b***). Mice undergoing topical application of chymostatin and leupeptin had an increased number of scales compared with vehicle-treated mice (**Fig 9*c***). Because protease inhibitors also induced scales

in vivo after topical application, the possibility that CS acts as an inhibitor in the stratum corneum is indicated.

DISCUSSION

The mechanism of regulation of desquamation in the stratum corneum is still unknown. We have shown previously that two types of serine protease are involved, and that degradation of desmosomes leads to desquamation (Suzuki *et al*, 1993, 1994). Lundström and Egelrud also reported chymotrypsin-like enzyme activity in the stratum corneum (Lundström and Egelrud, 1991; Sondell *et al*, 1995). The disorder of cornification, recessive X-linked ichthyosis, exhibits retention hyperkeratosis accompanied by accumulation of CS. Because CS inhibits the serine protease, acrosin (Burck and Zimmerman, 1980), we decided to examine the possibility that CS also plays a role as an inhibitor in desquamation.

Increased thickness of the stratum corneum reportedly is induced by topical application of CS without acanthosis, increased labeling index, or dermal inflammation (Maloney *et al*, 1984; Elias *et al*, 1984). We also observed scaling on the backs of hairless mice after topical applications of CS, and confirmed that there was no difference between CS-treated and vehicle-treated mice in the thickness of the epidermis and the labeling index with proliferating cell nuclear antigen, an index of proliferative activity. In contrast, the number of stratum corneum layers and the content of desmoglein I in the stratum corneum of CS-treated mice were higher than those in vehicle-treated mice. These results suggest that digestion of desmosomal proteins is inhibited by CS treatment.

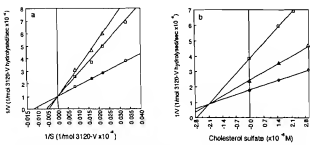

Figure 8. Cholesterol sulfate is a competitive inhibitor of chymotrypsin. (*a*) Lineweaver–Burk plot of the inhibition of chymotrypsin by cholesterol sulfate (△, 2.98 × 10^{-6} M; □, 2.23 × 10^{-6} M); ○, no cholesterol sulfate. (*b*) Dixon plot of chymotrypsin by cholesterol sulfate (○, 1.21 × 10^{-4} M 3120-V; △, 6.06 × 10^{-5} M 3120-V; □, 3.03 × 10^{-5} M 3120-V).

It has been reported that CS also acts as a second messenger for protein kinase C, hence the effects of CS on stratum corneum retention might be mediated indirectly (Chida *et al*, 1995). To avoid such effects of CS on living keratinocytes, we examined the effect of CS in the stratum corneum sheets. We found that the cell dissociation from the stratum corneum sheet was inhibited by CS, suggesting that CS acts directly on the cell shedding in the stratum corneum.

Finally, we examined the extent of the inhibitory properties of CS using commercially available crystallized trypsin and chymotrypsin as model enzymes, because enzymes in the stratum corneum were shown to be trypsin-like and chymotrypsin-like proteases (Suzuki *et al*, 1993, 1994). CS competitively inhibited both trypsin and chymotrypsin with K_i values of 5.5 × 10^{-5} M and 2.1 × 10^{-4} M, respectively. Thus, *in vitro* experiments for inhibitory properties of CS on model enzymes showed that K_i values had micromolar concentrations. We used 1–10 mM CS in the stratum corneum sheet assay and topical application, because there may be some difficulties with CS penetration into the stratum corneum. Maloney *et al* (1984) also reported that ≈5–10 mM of CS was needed to induce abnormal scales.

Although leupeptin and chymostatin showed more potent inhibitors of cell dissociation than CS in the stratum corneum sheet assay (data not shown), application of CS results in more obvious stratum corneum scaling compared with the mixture of leupeptin and chymostatin. This may reflect a high affinity of CS for the stratum corneum intercellular lipids, and for the physicochemical properties of CS itself (Williams, 1991).

To examine whether CS acts as a detergent to disrupt enzyme function, amphopatic lipids were tested in looking for scaling *in vivo*, the cell dissociation assay, and the enzyme assay *in vitro*. Topical application of phosphatidylcholine did not induce scales. The cell dissociation from the stratum corneum was not inhibited by phosphatidylcholine, palmitic acid, and taurocholic acid. In the enzyme assay system *in vitro*, phosphatidylcholine, palmitic acid, cholesterol, and taurocholic acid did not show inhibitory activity. These results show the effect is not simply a detergent effect.

The above results indicate that CS influences desquamation by acting as a serine protease inhibitor. Further, the accumulated stratum corneum in recessive X-linked ichthyosis may be caused by the inhibition of trypsin-like and chymotrypsin-like proteases by excessive CS. This could account for the abnormal persistence of numerous desmosomal structures in the outermost layers of the stratum corneum in this disease (Anton-Lamprecht, 1974; Bazex *et al*, 1978; Mesquita-Guimaraes, 1981). Our results suggest a possible new role of CS as an inhibitor in desquamation. In addition, quantitative lipid analysis of porcine

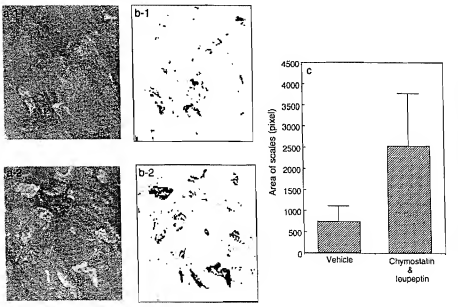

Figure 9. Increasing the abnormal scales on the backs of mice after topical application of chymostatin and leupeptin. Skin surface appearance of a normal vehicle-treated control mouse (*a-1*) *versus* a chymostatin and leupeptin treated mouse (*a-2*) and its binary images of a normal vehicle-treated control mouse (*b-1*) *versus* a chymostatin and leupeptin treated mouse (*b-2*). Scales were assessed by measurement of its area using a binary image and were quantitated by NIH image. Increased scales on mice by topical application of chymostatin and leupeptin in comparison with that from vehicle-treated mice (*c*) (n = 4, mean ± SD). Cholesterol sulfate *versus* control, $p < 0.05$.

epidermal strata revealed that CS exhibited its concentration in the deeper stratum corneum and then abruptly decreased in the surface layer (Cox and Squier, 1986). Thus, CS might regulate not only desquamation in pathologic stratum corneum, but also normal desquamation; the content of CS in the stratum corneum might influence the desquamation process in the normal stratum corneum.

The authors wish to express their gratitude to Prof. Dr P. M. Elias for helpful suggestions.

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